

1 **Genomic surveillance of *Clostridioides difficile* transmission and virulence in a healthcare setting**

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15 **Abstract**

16 *Clostridioides difficile* infection (CDI) is a major cause of healthcare-associated diarrhea,
17 despite the widespread implementation of contact precautions for patients with CDI. Here, we
18 investigate strain contamination in a hospital setting and genomic determinants of disease
19 outcomes. Across two wards over six months, we selectively cultured *C. difficile* from patients
20 (n=384) and their environments. Whole-genome sequencing (WGS) of 146 isolates revealed
21 that most *C. difficile* isolates were from clade 1 (131/146, 89.7%), while only one isolate of the
22 hypervirulent ST1 was recovered. Of culture-positive admissions, 17% of patients were
23 diagnosed with CDI upon admission. We defined 29 strain networks at ≤ 2 core gene SNPs; 2 of
24 these networks contain strains from different patients. Strain networks were temporally linked
25 ($p < 0.0001$). Across networks and over time, we found a minority of networks contained
26 differences in phage populations. To understand genomic correlates of disease, we conducted
27 WGS on an additional cohort of *C. difficile* (n=102 isolates) from the same hospital and
28 confirmed that clade 1 isolates are responsible for most CDI cases. We found that while
29 toxigenic *C. difficile* isolates are associated with the presence of *cdtR*, nontoxigenic isolates
30 have an increased abundance of prophages. Our pangenomic analysis of clade 1 isolates
31 suggests that while toxin genes (*tcdABER* and *cdtR*) were associated with CDI symptoms, they
32 are dispensable for patient colonization. These data indicate toxigenic and nontoxigenic *C.*
33 *difficile* contamination persists in a hospital setting and highlight further investigation into how
34 accessory genomic repertoires contribute to *C. difficile* colonization and disease.

35 Background

36 *Clostridioides difficile* infection (CDI) is one of the most common healthcare-associated
37 infections (HAIs) in the US and is the leading cause of healthcare-associated infectious
38 diarrhea^{1,2}. Since the early 2000s, *C. difficile* research has focused largely on hypervirulent
39 strains, such as PCR ribotype 027^{1,3-6}, which have been responsible for hospital-associated CDI
40 epidemics. Strains of ribotype 027 were responsible for 51% and 84% of CDI cases in the US
41 and Canada in 2005, respectively^{1,4,5}. Since then, other circulating strains have emerged as the
42 prevalent strains causative of CDI, such as 078 and 014/020⁷⁻⁹. One report indicated that the
43 prevalence of PCR ribotype 027 decreased from 26.2% in 2012 to 16.9% in 2016⁹. As the
44 landscape of *C. difficile* epidemiology continues to evolve, we must update our understanding of
45 how various strains of this pathogen evolve, spread, and cause disease.

46 In addition to the changing prevalence of CDI-causing *C. difficile* strains, their
47 transmission dynamics also appear to be evolving. In the late 1980s, it became clear that
48 patients with active CDI shed spores onto their surroundings, leading to future CDI events in the
49 healthcare setting¹. Because of this, patients with active CDI are placed on contact precautions
50 to prevent transmission to susceptible patients, which has been successful in reducing rates of
51 CDI^{2,10}. Nevertheless, while epidemiological estimates indicate that 20-42% of infections may be
52 connected to a previous infection, multiple genomic studies fail to associate a CDI case to a
53 previous case¹¹⁻¹³. This suggests other potential sources of pathogen exposure in the hospital
54 environment. While asymptomatic carriers of *C. difficile* have not been a significant focus of
55 infection prevention efforts, studies have shown these carriers do shed viable, toxigenic *C.*
56 *difficile* to their surroundings that could cause disease¹⁴. Several studies have shown evidence
57 of a reduction in CDI cases if asymptomatic carriers are put on similar contact precautions to
58 CDI patients¹⁵⁻¹⁷, but this has not been consistently found¹⁸. Correspondingly, it is critical to
59 understand if *C. difficile* carriers are major contributors to new *C. difficile* acquisition or CDI
60 manifestation in hospitalized patient populations.

61 *C. difficile* strains are categorized into five major clades and three additional cryptic
62 clades. These clades encompass immense pangenomic diversity with many mobilizable
63 chromosomal elements^{19,20}, including numerous temperate phages that have potential
64 influences over *C. difficile* toxin expression, sporulation, and metabolism²¹. Two major toxin loci,
65 not required for viability, encode large multi-unit toxins that independently augment the virulence
66 of *C. difficile*. Epithelial destruction and CDI have largely been attributed to the presence of
67 pathogenicity locus (PaLoc) encoding toxins TcdA and TcdB. In addition, an accessory set of
68 toxins (CdtA and CdtB) encoded at the binary toxin locus, may worsen disease symptoms²².

69 Yet, many nontoxigenic strains of *C. difficile* have been documented and are adept colonizers of
70 the GI tract, even without the PaLoc²³. As there has been continued debate about strain-specific
71 virulence attributes²⁴⁻²⁶, it is important to investigate the extent of strain-level pangenomic
72 diversity and consequences of such diversity on host disease^{27,28}.

73 The purpose of this study was to evaluate the role of *C. difficile* strain diversity in
74 colonization outcomes and hospital epidemiology. By sampling patients (n=384) and their
75 environments for six months in two leukemia and hematopoietic stem cell (HCT) transplant
76 wards at Barnes-Jewish Hospital in St. Louis, USA, we used isolate genomics to identify
77 environmental contamination of both toxigenic (TCD) and nontoxigenic (NTCD) *C. difficile* by
78 carriers and CDI patients, and corresponding transmission between both patient groups.
79 Longitudinal strain tracking within these transmission networks revealed accessory gene flux of
80 multi-drug resistance loci over the course of the study. Lastly, integration of isolate genomic
81 data and CDI information from this prospective study with isolate genomic data from a
82 complementary retrospective study of asymptomatic vs symptomatic *C. difficile* colonization in
83 the same hospital^{29,30} indicated that the clade 1 lineage, containing both toxigenic strains and
84 nontoxigenic strains, dominates circulating populations of *C. difficile* in this hospital. Further, this
85 lineage of *C. difficile* has significant variation in the PaLoc operon, and harbors other genetic
86 factors that are associated with CDI symptoms in patients.

87 **Methods**

88 Study Design

89 This prospective observational study took place in the leukemia and hematopoietic stem cell
90 transplant (HCT) wards at Barnes-Jewish Hospital (BJH) in St. Louis, Missouri, United States.
91 Each ward consisted of two wings with 16 beds; on the acute leukemia ward we enrolled from
92 both wings (32 beds) and on the HCT ward we enrolled on one wing (16 beds). The wards were
93 sampled for 6 months from January 2019-July 2019 (acute leukemia) and 4 months from March
94 2019-July 2019 (HCT). These units are located 2 floors apart in the same building.

95 Sample collection, selective culture, and isolate identification

96 Patients and their environments were sampled upon admission to a study ward and then weekly
97 until discharge. Per hospital standards, bleach is used for daily and terminal discharge cleaning.
98 From each patient, a stool specimen and/or rectal swab was collected as available. Remnant
99 fecal samples from the BJH microbiology laboratory that were obtained during routine clinical
100 care were also collected. Stool samples and rectal swabs collected on enrollment were
101 refrigerated for up to 3 hours before processing. Specimens from all other timepoints were

102 stored in at -80°C in tryptic soy broth (TSB)/glycerol before processing. Environmental samples
103 were collected from bedrails, keyboards, and sink surfaces using 3 E-swabs (Copan). If a
104 surface was unable to be sampled, a swab was taken from the IV pump or nurse call button as
105 an alternative. Swab eluate were stored at -80°C until processing.

106 Broth enrichment culture for *C. difficile* in Cycloserine Cefoxitin Mannitol Broth with
107 Taurocholate and Lysozyme (CCMB-TAL) was performed on all admission specimens and
108 checked for growth at 24h, 48h, and 7 days after inoculation. If that culture produced *C. difficile*,
109 all other specimens collected from that patient and their surroundings were also cultured on
110 Cycloserine-Cefoxitin Fructose Agar with Horse Blood and Taurocholate (CCFA-HT) agar.
111 Colonies resembling *C. difficile* (large, spreading, grey, ground glass appearance) were picked
112 by a trained microbiologist and sub-cultured onto a blood agar plate (BAP). Growth from the
113 subculture plate was identified using Matrix-assisted laser desorption/ionization-time of flight
114 mass spectrometry (MALDI-TOF MS). Upon identification, sweeps of *C. difficile* BAPs were
115 collected in tryptic soy broth (TSB) and stored at -80C for sequencing. If both rectal swab
116 sample and stool sample produced a *C. difficile* isolate, the stool isolate was preferentially used
117 for analysis over the rectal swab isolate.

118 *C. difficile* toxin enzyme immunoassay (EIA) was conducted as part of routine clinical care
119 based on clinical suspicion of CDI. To be diagnosed with *C. difficile* infection (CDI), a patient
120 must have been EIA+ for *C. difficile* toxin (Alere TOX A/B II); those who weren't tested (due to
121 no clinically significant diarrhea) or tested EIA- and were culture-positive for *C. difficile* were
122 considered *C. difficile* carriers. Episodes of carriage or CDI are defined as the time from the first
123 culture-positive specimen from a patient to the last culture-positive specimen during a given
124 hospital admission.

125

126 Short read sequencing and *de novo* genome assembly

127 Parameters used for computational tools are provided parenthetically. Total genomic DNA from
128 *C. difficile* isolates was extracted from frozen plate scrapes using the QIAamp BiOstic
129 Bacteremia DNA Kit (Qiagen) and quantified DNA with the PicoGreen dsDNA assay (Thermo
130 Fisher Scientific). DNA from each isolate was diluted to a concentration of 0.5 ng/μL for library
131 preparation using a modified Nextera kit (Illumina) protocol³¹. Sequencing libraries were pooled
132 and sequenced on the NovaSeq 6000 platform (Illumina) to obtain 2x150bp reads. Raw
133 reads were demultiplexed by index pair and adapter sequencing trimmed and quality filtered
134 using Trimmomatic (v0.38, SLIDINGWINDOW:4:20, LEADING:10, TRAILING:10,

135 MINLEN:60)³². Cleaned reads were assembled into draft genomes using Unicycler (v0.4.7)³³.
136 Draft genome quality was assessed using Quast³⁴, BBSMap³⁵, and CheckM³⁶, and genomes
137 were accepted if they met the following quality standards: completeness greater than 90%,
138 contamination less than 5%, N50 greater than 10,000 bp, and less than 500 contigs >1000bp.

139 Isolate characterization and typing

140 A Mash Screen was used to identify likely related genomes from all NCBI reference genomes³⁷.
141 Average nucleotide identity(ANI) between the top three hits and the draft assembly was
142 calculated using dnadiff³⁸. Species were determined if an isolate had >75% alignment and
143 >96% ANI³⁹ to a type strain, and were otherwise classified as genomospecies of the genus level
144 taxonomy call.

145 In silico multilocus sequence typing (MLST) was determined for all *C. difficile* and
146 genomospecies isolates using mlst^{40,41}. Isolate contigs were annotated using Prokka⁴² (v1.14.5,
147 -mincontiglen 500, -force, -rnammer, -proteins GCF_000210435.1_ASM21043v1_protein.faa⁴³).
148 *cdtAB* was determined to be a pseudogene if there were three hits to *cdtB*, indicating the
149 damaged structure of the pseudogene⁴⁴. *C. difficile* clade was determined using predefined
150 clade-MLST relationships described in Knight, et al¹⁹.

151 Phylogenetic analyses

152 The .gff files output by Prokka⁴² were used as input for Panaroo (v1.2.10)⁴⁵ to construct a core
153 genome alignment. The Panaroo alignment was used as input to construct a maximum-
154 likelihood phylogenetic tree using Fasttree⁴⁶. The output .newick file was visualized using the
155 ggtree (v3.4.0)⁴⁷ package in R. Cryptic clade isolates were determined as such based on
156 phylogenetic clustering with cryptic clade reference isolates.

157 Core genome SNP analyses and network formation

158 We constructed a core gene alignment for each clade using Panaroo (v1.2.10) and calling
159 MAFFT (v7.481). We then used Gubbins (v3.3.0) to identify recombination-filtered polymorphic
160 sites, and constructed a recombination-free polymorphic site alignment using snp-sites (v2.4.0
161 25414349)⁴⁸. We finally extracted pairwise, recombination-filtered clade specific core-gene SNP
162 distances using snp-dists (v0.8.2)(<https://github.com/tseemann/snp-dists>). Strain networks were
163 determined by connecting isolates that were <=2 SNPs from one another.

164 Phage identification and clustering

165 Isolate genomes were piped into Cenote-Taker 2⁴⁹ to identify contigs with end features as direct
166 terminal repeats (DTRs) indicating circularity and inverted linear repeats (ITRs) or no features
167 for linear sequences. Identified contigs were filtered by length and completeness to remove
168 false positives. Length limits were 1,000 nucleotides (nt) for the detection of circularity, 4,000 nt
169 for ITRs, and 5,000 nt for other linear sequences. The completeness was computed as a ratio
170 between the length of our phage sequence and the length of matched reference genomes by
171 CheckV⁵⁰ and the threshold was set to 10.0%. Phage contigs passing these two filters were
172 then run through VIBRANT⁵¹ with a “virome” flag to further remove obvious non-viral
173 sequences⁵¹. Based on MIUViG recommended parameters⁵², phages were grouped into
174 “populations” if they shared $\geq 95\%$ nucleotide identity across $\geq 85\%$ of the genome using
175 BLASTN and a CheckV supporting code.

176 Analysis of genotypic associations with disease severity

177 Two previously sequenced retrospective cohorts from the same hospital were included to
178 increase statistical power^{29,53}. In the analyses of toxigenic vs. nontoxigenic isolates from clade
179 1, Pyseer⁵⁴ was run using a SNP distance matrix (using snp-dist as above), binary
180 genotypes (presence or absence of *tcdB*), and Panaroo-derived gene presence/absence data.
181 In the analysis of CDI suspicion, all isolates from clade 1 were used that represented one isolate
182 per patient-episode. Isolates recovered from environmental surfaces were excluded. Using
183 these assemblies, a core genome alignment was generated using Prokka⁴² and Panaroo⁴⁵ as
184 above. SNP distances were inferred from the core-gene alignment using snp-dists⁵⁵. Binary
185 phenotypes were coded for the variable CDI suspicion, whereby isolates associated with a
186 clinically tested stool were associated with symptomatic colonization (TRUE). Isolates that were
187 associated with a surveillance stool and had no clinical testing associated with that patient
188 timepoint were coded as non-symptomatic colonization (FALSE). Gene candidates filtered
189 based on ‘high-bse’, and were annotated HMMER on RefSeq databases and using a
190 bacteriophage-specific tool VIBRANT⁵¹. Selected outputs were visualized in R using the beta
191 coefficient as the x-axis and the $-\log_{10}$ (likelihood ratio test p-value) as the y-axis.

192 Reference assembly collection

193 We chose 23 reference assemblies from Knight, et al¹⁹ for Figure 2c because of their MLST-
194 clade associations (Supplementary Table 2). References span Clades 1-5 and cryptic clades C-
195 1, C-2, and C-3, with one reference from each of the three most frequent MLSTs in each clade.
196 Cryptic clade C-3 only had 2 reference assemblies available. References were annotated and
197 included in phylogenetic tree construction as above.

198 All *Clostridioides difficile* genomes available on the National Institutes of Health (NIH) National
199 Library of Medicine (NLM) were acquired for Figure 5c construction. References from NCBI
200 (Supplementary Table 4) were included if they had less than 200 contigs. Assemblies that met
201 these quality requirements were annotated and phylogenetically clustered as above.

202 **Results**

203 Surveillance of *C. difficile* reservoirs in hospital wards reveals patient colonization and 204 environmental contamination.

205 We prospectively collected patient and environmental samples to investigate genomic
206 determinants of *C. difficile* carriage, transmission, and CDI (Figure 1). Across the study period,
207 we enrolled 384 patients from 654 unique hospital admissions, and collected patient specimens
208 upon admission and weekly thereafter (Supplementary Figure 1). We collected at least one
209 specimen (clinical stool collected as part of routine care, study collected stool, or study collected
210 rectal swab) from 364 admissions (94.8% of enrolled patients), for a total of 1244 patient
211 specimens. We selectively cultured *C. difficile* from 43 rectal swabs and 108 stool samples, for a
212 total of 151 culture-positive patient specimens. We also collected weekly swabs from the
213 bedrails, sink surfaces, and in-room keyboards, for a total of 3045 swabs from each site. In total,
214 22/398 (5.5%) of bedrail swabs cultured and 4/ 399 (1.0%) of keyboard swabs cultured were
215 culture-positive for *C. difficile* (Figure 2a). *C. difficile* was never recovered from sink surfaces (all
216 sinks on these units are hands-less activated) or other sampled sites. Collapsing multiple
217 positive samples from the same patient admission results in 20 positive bedrails (20/79, 25.3%
218 of all admissions with positive patient specimens) and 4 positive keyboards (4/79, 5.06% of all
219 admissions with positive patient specimens) (Figure 2b).

220 *C. difficile* carriers outnumbered patients with CDI

221 Patients with CDI were identified through routine clinical care, with CDI defined as
222 patients who had stool submitted for *C. difficile* testing, as ordered by the clinical team when
223 suspicious for CDI, and who tested positive for *C. difficile* toxins by enzyme immunoassay
224 (EIA+). Otherwise, if they were culture positive and EIA- or culture positive and not EIA tested,
225 they were considered carriers. Results from selective culture indicated that 21.7% of unique
226 admissions (79/364 admissions with available specimens) were culture-positive for *C. difficile* at
227 some point during their admission (Figure 2b). Of culture-positive admissions, 17% (13/79) were
228 EIA+ and diagnosed with CDI (13/364, 3.6% of all admissions with specimens available). The
229 remaining 83% (66/79 admissions with specimens available) of culture-positive admissions

230 were termed carriers (Figure 2b). An additional nine admissions became EIA+ at some point
231 during their stay for a total of 22 CDI cases, but seven did not have specimens available for
232 culture and two were culture negative. The substantial detection of longitudinal patient *C.*
233 *difficile* colonization prompted us to investigate the genomic correlates of *C. difficile*-associated
234 disease and transmission in these two patient populations.

235 Phylogenetic clustering reveals lack of hypervirulent strains, presence of cryptic clades

236 We conducted whole-genome sequencing to ascertain phylogenetic distances among isolates
237 and to identify closely related strains of *C. difficile*. We identified 141 isolate genomes as *C.*
238 *difficile* (using a 75% alignment and 96% average nucleotide identity [ANI] threshold). One
239 isolate was identified as *Clostridium innocuum* and five isolates were classified as *C. difficile*
240 genomospecies (92-93% ANI). To contextualize population structure, we applied a previously
241 established MLST-derived clade definition to our isolate cohort¹⁹. The majority of *C. difficile*
242 isolates were from Clade 1 (131/146, 89.7% of *C. difficile* and genomospecies, Figure 2c). Four
243 patient-derived isolates were identified from clade 2, but only one was of the hypervirulent strain
244 ST1 (PCR ribotype 027)⁶. We found that the distribution of STs associated with carriers was
245 significantly different from that of STs associated with CDI patients ($p < 0.001$, Fisher's exact
246 test) suggesting some strain-specificity to disease outcome.

247 Interestingly, the five genomospecies isolates clustered with other isolates belonging to
248 a recently discovered *C. difficile* cryptic clade C-1 (Supplementary Figure 2). While cryptic
249 clades are genomically divergent from *C. difficile*, these isolates can produce homologs to
250 TcdA/B and cause CDI-like disease in humans^{19,56}. In a clinical setting, they are frequently
251 identified by MALDI-TOF MS as *C. difficile* and diagnosed as causative of CDI⁵⁶. These data
252 highlight the novel distribution of circulating *C. difficile* strains in the two study wards. While
253 many patients with multiple isolates had homogeneous signatures of colonization (with closely
254 related isolates), four patients (4/72, 6%) produced isolates from distinct ST types.

255 Carriers and CDI patients contribute to transmission networks and environmental contamination

256 Given the predominance of Clade 1 isolates, we sought to identify clonal populations of *C.*
257 *difficile* strains, indicative of direct *C. difficile* contamination (patient-environment) or
258 transmission (patient-patient). We compared pairwise, recombination-filtered within-clade core
259 gene single nucleotide polymorphism (SNP) distances to identify networks of transmission
260 connecting isolates ≤ 2 SNPs apart (Supplementary Figure 4). We identified a total of 29 strain
261 networks, 2 of which contain patient isolates from different patients (Figure 3a). These strain

262 networks were temporally linked, as there were significantly fewer days between same-network
263 isolates than isolates from different networks ($p < 2.2e-16$, Wilcoxon, Figure 3b). We compared
264 strain connections among a single patient's isolates from stool or rectal swab ('patient'), and
265 between these isolates and environmental isolates from their immediate surroundings ('bedrail'
266 or 'keyboard', Figure 3c). While the majority of bedrail isolates fell within the same network as
267 patient isolates from that room (30 of 44 comparisons, 68%), 32% (14 of 44 comparisons) were
268 genomically distinct, suggesting contamination from alternate sources. Keyboards were mostly
269 colonized with distinct strains from the patient (22%, 2/9 comparisons were the same strain),
270 indicating other routes of contamination ($p < 0.05$, Fisher's exact test, BH corrected. Figure 3c).
271 Among the networks that contain multiple patients, we found no instances of potential
272 transmission from the inhabitant of one room to the subsequent inhabitant. However, in both
273 instances, each potential transmission was associated with a temporal overlap in patient stay in
274 the same ward, providing epidemiological capacity for transmission ($p < 0.05$, Wilcoxon test).
275 Importantly, we found no networks connecting patients with CDI to *C. difficile* carriers,
276 suggesting successful containment through contact precaution protocols. These data highlight
277 multiple sources of environmental contamination by *C. difficile* and prompted us to investigate
278 the relationship between genetic factors and patient symptomology.

279 Phage populations persist in circulating *C. difficile* networks

280 *C. difficile* isolates have an extensive pangenome, with genetic loci mobilized by
281 conjugative elements and phages, and mobilizable elements playing a key role in *C. difficile*'s
282 lifecycle⁵⁷. Temperate phages, which can undergo lytic replication or insert into the host genome
283 as a latent prophage, are the only phages that have been isolated for *C. difficile*⁵⁸. To identify *C.*
284 *difficile* prophage signatures and understand how dynamic they were in our strain networks, we
285 analyzed our isolate genomes with Cenote-Taker 2 for putative phage contigs. After filtering for
286 quality, we grouped contigs into phage populations (vOTUs) and quantified the alpha-diversity
287 of phage populations in each isolate, and across MLST types (Figure 4a). ST42 and ST2, some
288 of the most globally abundant ST types had the lowest diversity of phages in our cohort, though
289 this negative correlation was not statistically significant across ST types (Figure 4b; $R = -0.31$,
290 $p = 0.12$). Our clonality-resolved strain networks allowed us to investigate phage flux over time.
291 We found that the majority of networks (23/29) carried the same number of phages over time
292 (Figure 4c), suggesting persistent roles in *C. difficile* biology. Interestingly, we found that
293 nontoxigenic isolates had a higher diversity of phage populations relative to toxigenic isolates

294 (Figure 4d). These data suggest distinct selective pressures on temperate phages in *C. difficile*
295 related to toxin gene presence.

296 Accessory genomic elements are associated with host CDI symptoms

297 Despite evidence of transmission in this prospective study, a minority of patients were
298 diagnosed with CDI relative to those asymptotically colonized with *C. difficile* in part due to
299 the presence of nontoxigenic *C. difficile* isolates (Figure 2b). To power our investigation of
300 virulence determinants across patient-colonizing *C. difficile* strains, we performed whole
301 genome sequencing on 102 additional patient-derived *C. difficile* isolates from a previously
302 described *C. difficile*-colonized/CDI cohort from the same hospital²⁹, where all patients had
303 clinical suspicion of CDI (CDI suspicion), defined by a clinician ordering an EIA test during
304 patient admission. Using an MLST-based clade definition as above, we identified that most CDI
305 cases result from isolates within clade 1, though clade 2 isolates were more likely to be
306 associated with CDI status (Figure 5a). The latter finding supports previous data indicating that
307 clade 2 isolates are hypervirulent, often attributed to the presence of the binary toxin operon or
308 increased expression from the PaLoc^{22,59,60}. Meanwhile, some clade 1 isolates contain no
309 toxins, indicating a diversity of colonization strategies in this lineage. Pangenomic comparison of
310 nontoxigenic versus toxigenic isolates revealed that in addition to the PaLoc, the majority of our
311 toxigenic isolates from clade 1 (95/131 of our cohort) possess remnants of the binary toxin
312 operon (Figure 5b, *cdtR* and *cdtA/B* pseudogenes). Given the previous report that full-length
313 *cdtAB* was identified only within Clades 2, 3, and 5¹⁹, we investigated the conservation of *cdtR*
314 (the transcriptional regulator of the binary toxin locus) across *C. difficile* strains (containing 5
315 lineages). We additionally examined >1400 *C. difficile* genome assemblies from NCBI
316 (Supplementary Table 4, Figure 5c). *cdtR* (unlike *cdtAB*) was dispersed across clade 1 and
317 significantly associated with *tcdB* (Figure 5d, Fisher's exact test, BH corrected), suggesting a
318 selective pressure to maintain some element of both toxin loci in these isolates. Notably, these
319 operons are not syntenic, further underlining the significance of the association. From this
320 association, we sought to further understand why some toxigenic clade 1 isolates cause CDI
321 and some colonize without symptoms. Using 148 toxigenic clade 1 isolates collected from this
322 study and two previous studies from the same hospital^{29,53}, we utilized a bacterial GWAS
323 approach, *pyseer*⁵⁴, that identifies genetic traits associated with strains corresponding to
324 patients with CDI symptoms. Using CDI suspicion (see Methods) as an outcome variable, we
325 found that, multiple amidases (including *cwiD*), putative transcriptional regulators, and many
326 genes of unknown function were enriched in isolates associated with CDI symptoms (Figure

327 5e). These data indicate that the most prevalent, circulating *Cd* strains that cause CDI are not
328 the hypervirulent clade 2 strains, but highlight the possibility that remnant genomic features from
329 epidemic strains and other features may contribute to virulence in this hospital clade of *C.*
330 *difficile*.

331 **Discussion**

332 Through our prospective genomics study of two hospital wards, we were able to identify
333 connections between the contamination of different surfaces and the strains carried by
334 hospitalized patients and quantify some spread between carriers. Our estimates of the
335 prevalence of patients with CDI (3.8%) agree with other estimates of 2-4% CDI in patients with
336 cancer⁶¹⁻⁶³. While many studies have quantified surface contamination, few have had the
337 genomic resolution to identify clonality between isolates indicating transmission or patient
338 shedding⁶⁴⁻⁶⁶. We observed distinct patterns of contamination between a patient's bedrail and
339 the corresponding room keyboard, supporting the notion that the bedrail could be one of
340 multiple critical points of transmission in a hospital setting. Further, we did not identify any
341 instances of CDI that could be genomically linked to an earlier CDI case or *C. difficile* carrier.
342 Despite the small sample size, these data support the continued use of contact precautions for
343 CDI patients¹⁸.

344 Our data suggests the need to continually update our understanding of CDI-causing *C.*
345 *difficile* strains beyond previous epidemic strains to clarify mechanisms of how the most
346 prevalent strains relate to transmission and disease. Across 146 patient specimens, we only
347 identified one incidence of the epidemic ST1 strain. This ribotype caused one case of CDI within
348 our cohort, corroborating the decline in this epidemic lineage⁶⁷. Because the overall burden of
349 Clade 1 isolates was so high, we hypothesize that its ability to colonize without causing CDI
350 could allow for a substantial expansion of transmission networks (especially for the case of
351 nontoxigenic strains). While Clade 1 isolates associated with CDI symptoms are expectedly
352 toxigenic (containing the toxin genes in the PaLoc), we also found an enrichment in two different
353 amidase genes, that could either contribute to differences in germination rate or possess
354 endolysin function^{68 69}. How the function of such a gene contributes to an increase in
355 symptomology remains to be understood. Further, we confirmed a genetic relationship between
356 *cdtR* and *tcdB* across *C. difficile* lineages that indicates some evolutionary pressure for
357 maintaining the regulatory gene of the less prevalent toxin operon (*cdtR*). This phylogenomic
358 analysis supports recent functional data from clade 2 isolates, where the presence of full-length
359 *cdtR* increases the expression of *tcdB* and disease severity in an animal model of CDI⁵⁷. While

360 this was previously suggested *in vitro*, it is unclear how generalizable this relationship is across
361 lineages⁵⁹. In fact, we predict that clade 1 isolates containing only *cdtR* and the PaLoc may
362 produce more toxin *in vivo*. Future studies are warranted to investigate the role of both classes
363 of genes implicated in this phenotype.

364 Our study contextualizes the need for investigating *C. difficile* evolution within patients
365 over time, especially concerning functional mobile units such as temperate phages. We
366 examined phage populations in our isolates as they are a relevant mobile unit of the *C. difficile*
367 pangenome and their stability over time has not been systematically investigated. While we find
368 that the majority of *C. difficile* strains maintain their diversity of phage populations over time, we
369 acknowledge that hospital admission is a prescribed period of time and we may be
370 underestimating the amount that phage diversity changes in isolates over longer periods of time
371 *in vivo*. Our quantification of increased phage diversity in nontoxicogenic isolates suggests phage
372 niche specialization based on the presence of the PaLoc. It is noteworthy that early
373 characterization of the PaLoc operon indicated that it was integrated into the *C. difficile*
374 chromosome by an ancient prophage⁷⁰⁻⁷². Future work is required to understand how persistent
375 phages function during *C. difficile* growth and pathogenesis⁵⁸.

376 Our study has a number of important limitations. As this study focused on *C. difficile*
377 colonization, disease, and transmission in two wards in the same hospital, studies with
378 increased sample size or meta-analysis studies are necessary to understand generalizable
379 epidemiological measurements of *C. difficile*-patient dynamics⁷³. Additionally, our study protocol
380 allowed for culturing all environmental/patient specimens from a carrier or patient with CDI.
381 Thus, it is possible that our estimate of carriage in this study population is an overestimate.
382 Finally, we note the evidence for multi-strain colonization within a single patient (Patient 2330).
383 Given our approach of only culturing and sequencing single isolates per patient timepoint, future
384 studies are needed to investigate the extent of within-patient *C. difficile* strain diversity by
385 interrogating additional cultured isolates per samples⁷⁴ or via metagenomic methods.

386 Despite these limitations, this work allows us to understand an updated genomic picture
387 of circulating *C. difficile* in hospital-associated patients: how strains spread, their evolution, and
388 their virulence potential in this study population. Indeed, though much human and animal
389 research has focused on epidemic strains that are two decades old, we and others have
390 identified more disease and colonization from distinct lineages of *C. difficile*, namely clade 1
391 lineages. Moreover, within this lineage we found a mosaic representation of the PaLoc that
392 highlighted the possibility of different mechanisms of colonization and virulence by this

393 population of *C. difficile*. Future studies utilizing other human cohorts or animal models are
394 warranted to investigate disease and pathogenicity caused by Clade 1 *C. difficile* strains.

395

396 **List of Abbreviations**

397 BAP: blood agar plate

398 CCFA-HT: Cycloserine-Cefoxitin Fructose Agar with Horse Blood and Taurocholate

399 CCMB-TAL: Cycloserine Cefoxitin Mannitol Broth with Taurocholate and Lysozyme

400 CDI: *Clostridioides difficile* infection

401 EIA: enzyme immunoassay

402 HAI: healthcare-associated infection

403 HGT: horizontal gene transfer

404 MALDI-TOF MS: Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

405 NTCD: non-toxigenic *C. difficile*

406 PaLoc: pathogenicity locus

407 TCD: toxigenic *C. difficile*

408 TSB: tryptic soy broth

409

410 **Declarations**

411 **Ethics approval and consent to participate**

412 The study protocol was approved by the Washington University Human Research Protection
413 Office (IRB #201810103). All participants provided written informed consent.

414 **Consent for publication**

415 Not applicable.

416 **Availability of data and materials**

417 The datasets generated and analyzed during the current study are available in NCBI GenBank
418 under BioProject accession no. PRJNA980715.

419 **Competing interests**

420 The authors declare that they have no competing interests.

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430 **Authors' contributions**

431 SRSF, KAR, ERD, and GD participated in idea formulation and funding for this project. TH,
432 KAR, CC, ZHI, ELS, and ERD conducted participant enrollment, sample collection, and
433 microbiological isolation. EPN, SRSF, KZ, and GD conducted all sequencing analysis and figure
434 generation. EPN and SRSF completed the writing of the manuscript. All authors read and
435 approved the final manuscript.

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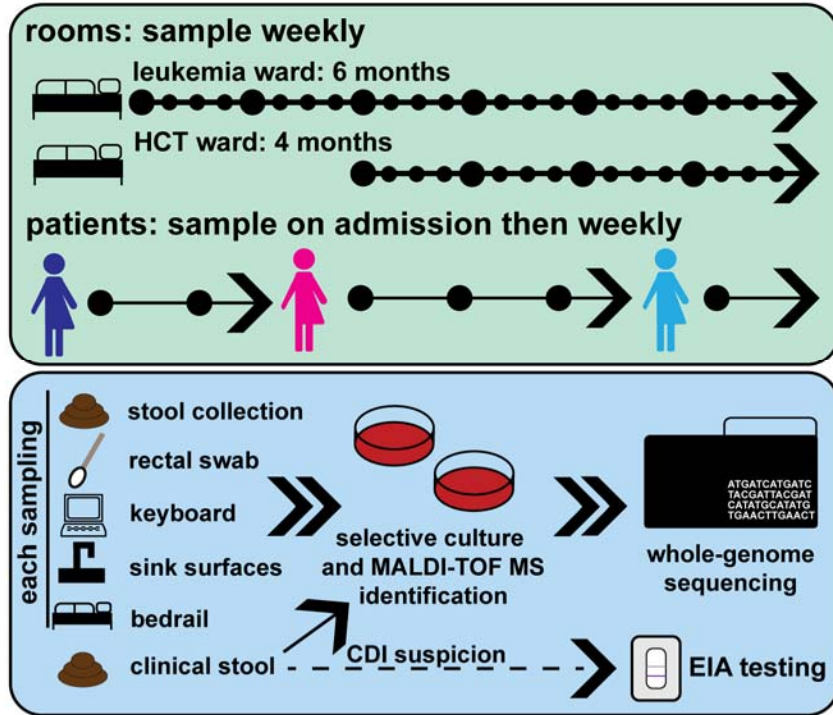
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648 **Figure Titles and Captions**



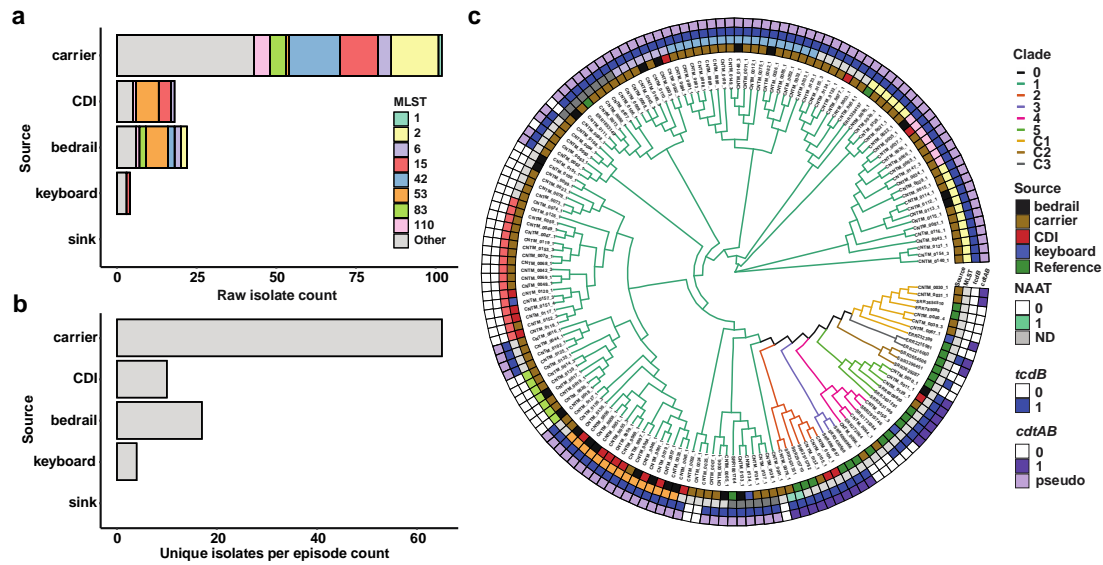
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650 Figure 1: Study sampling and testing overview.

651 Caption: a) We sampled a leukemia and hematopoietic stem cell transplant ward at Barnes-
652 Jewish Hospital in St. Louis, USA for 6 and 4 months respectively. Patients were enrolled and
653 sampled upon admission, and then weekly for their time in the study wards. Surfaces were
654 sampled weekly across the duration of the study. All samples and stool collected as part of
655 routine clinical care were subjected to selective culture and MALDI-TOF MS identification, and
656 isolates were whole-genome sequenced. Results of EIA testing as part of routine care were
657 obtained.

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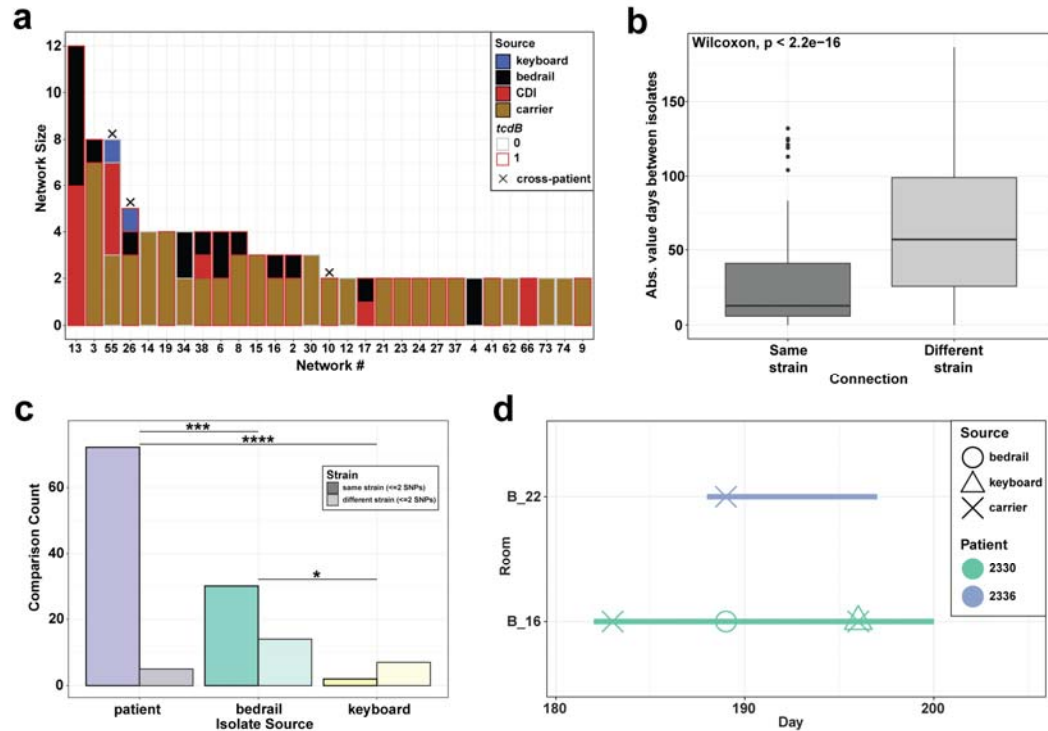
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661 Figure 2: Total samples collected and phylogenetic relationships reveal carriers outnumber CDI
662 patients and bedrails are the most commonly contaminated surface.

663 Caption: Total a) isolates collected and b) culture-positive episodes from each source. We found
664 more carriers than CDI patients, and bedrails yielded the most *C. difficile* isolates. c) Cladogram
665 of all isolates collected during this study plus references.

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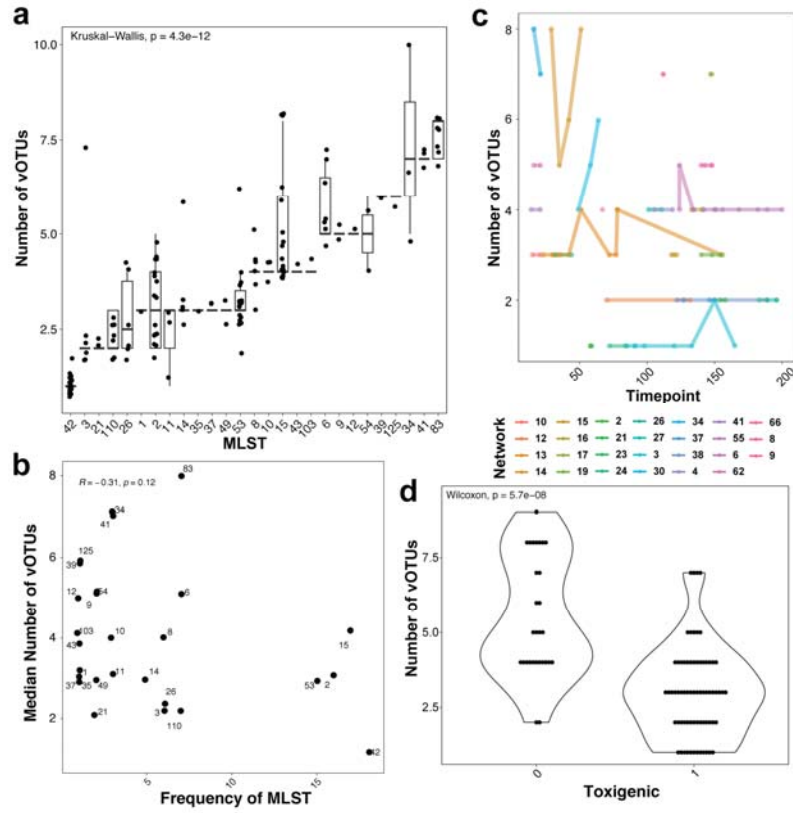
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Figure 3: Hospital bedrails are a site of environmental contamination from colonized and CDI patients.

Caption: a) Strain networks were defined by ≤ 2 core gene SNP cutoff. Network 55 includes the non-toxigenic isolates from Patient 2245 that are likely not responsible for the CDI. b) Absolute value of days between isolates within strains and between strains. Isolates within the same strain were significantly temporally linked ($p < 2.2e-16$, Wilcoxon test). c) Number of comparisons in each group that fall within strain cutoff. Fisher's exact test, BH corrected. d) Strain tracking diagram of transmission network 26, colors indicate patients and horizontal lines indicate stay in a room. Patient 2336 sheds *C. difficile* onto the bedrail in room B_16, and patient 2330 later is identified as a carrier of the same strain.



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683 Figure 4: Phage persistence in circulating *C. difficile* networks.

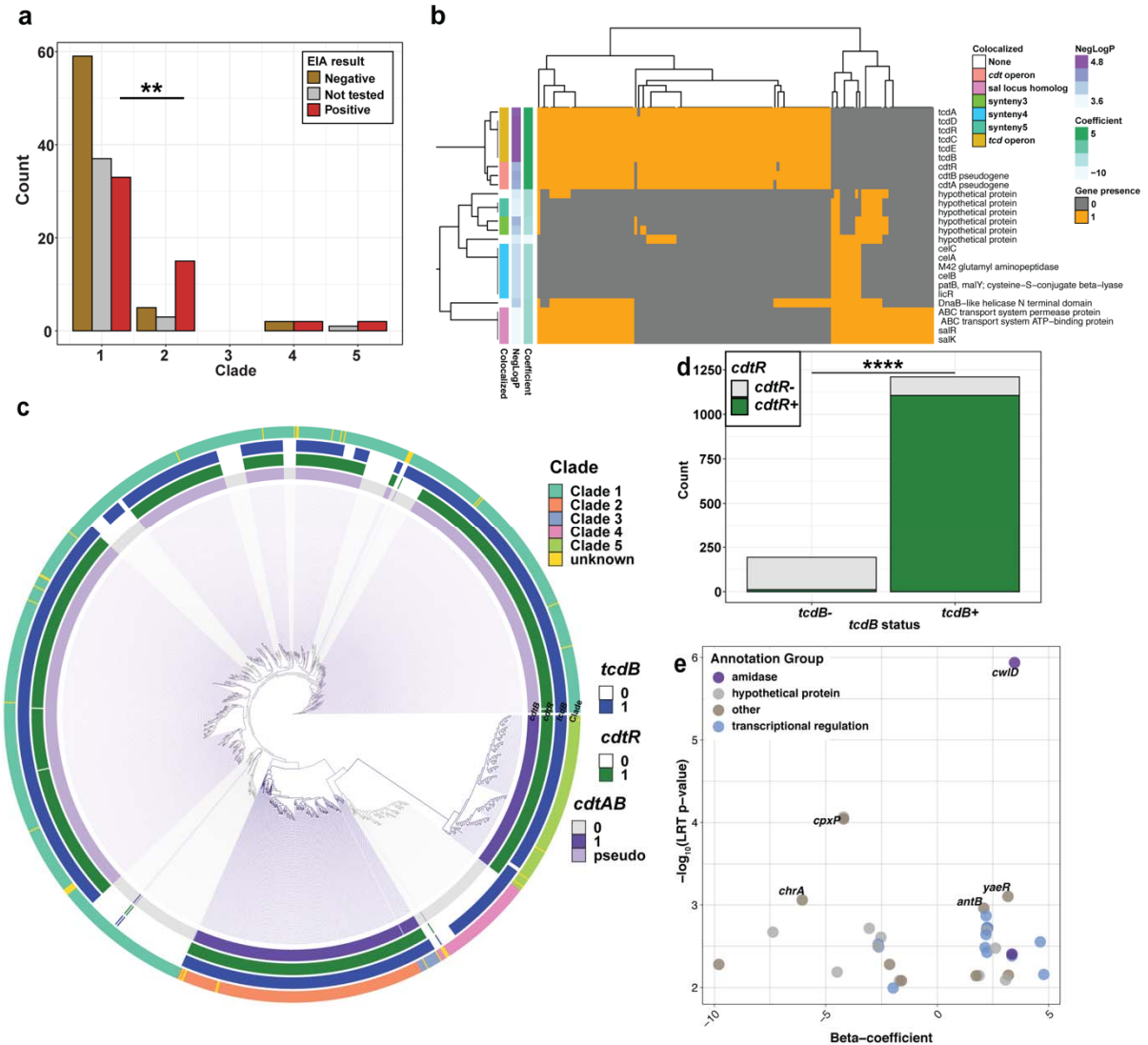
684 Caption: a) Phage diversity measured by phage population abundance for each isolate within an

685 MLST. b) Relationship between phage diversity and frequency of ST in our cohort c) Temporal

686 trajectory of phage diversity for each network over time. d) phage population richness across

687 toxigenic and nontoxigenic isolates in our cohort, Wilcoxon test, $p < 0.001$.

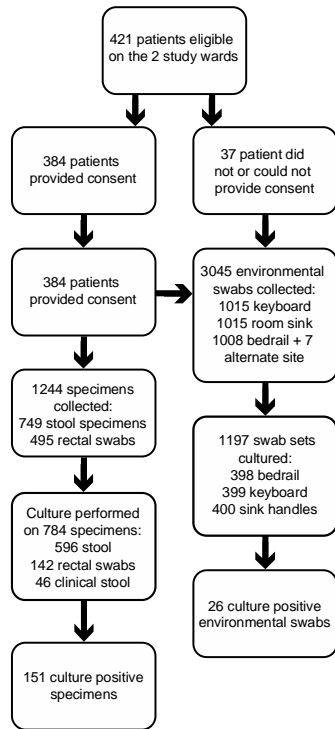
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 690 Figure 5: Clade 1 is responsible for the majority of CDI cases and carries unique correlates to
 691 symptom severity.
 692 Caption: a) EIA status by clade across this and a previous study²⁹. Fisher’s exact test, $p < 0.01$.
 693 c) Phylogenetic tree of >1400 *C. difficile* isolates from NCBI (Supplementary Table 4) depicting
 694 presence of binary toxin and PaLoc operons. d) Presence of full-length *cdtR* and association
 695 with *tcdB* presence. e) Filtered results (p -values < 0.01) pyseer analysis evaluating gene
 696 association with CDI suspicion in Clade 1 isolates using the phylogenetically-corrected p -values
 697 (LRT). Purple color indicates $p < 0.001$. Positive beta coefficient indicates gene association with
 698 CDI suspicion, while negative beta indicates asymptomatic colonization.

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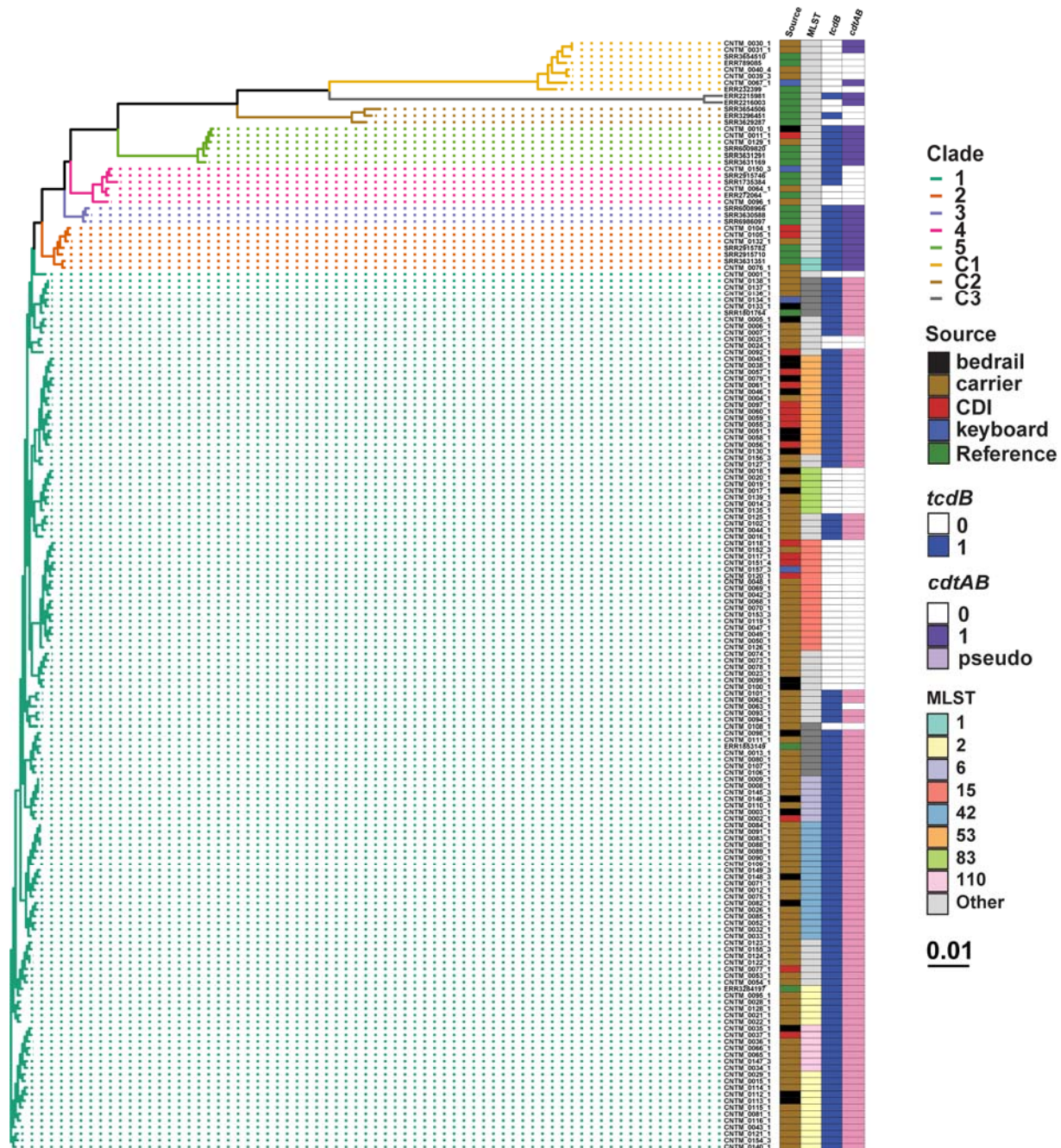
702 **Supplemental Figures and Tables Titles and Captions**

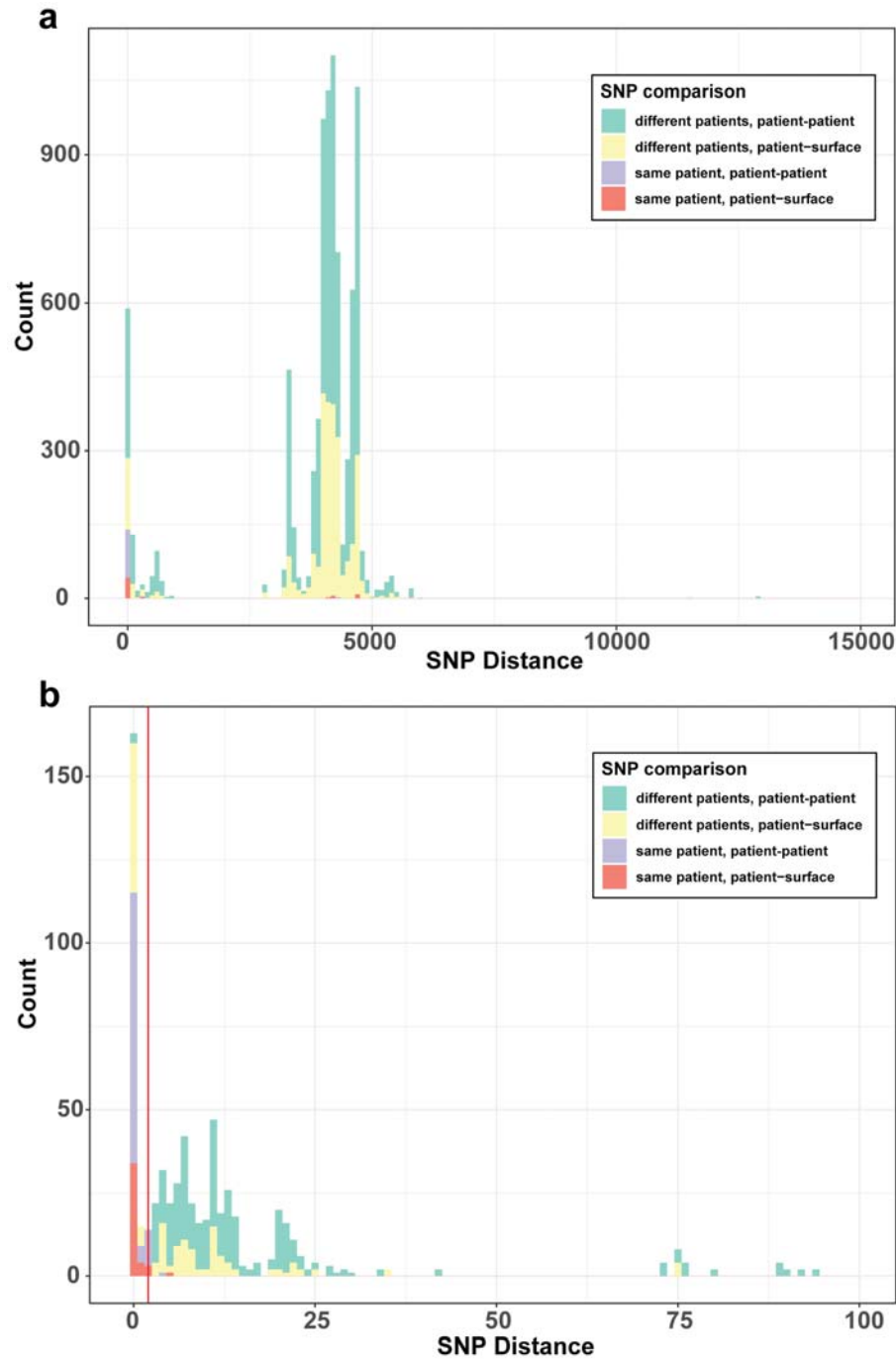


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704 **Supplementary Figure 1: Bubble plot of enrollment, collection, and culture numbers.**

705
 706 Supplementary Figure 2: Phylogenetic tree of isolates collected in this study and select
 707 references (Supplementary Table 2).





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709 Supplementary Figure 3: Histogram of core genome SNP distances between different isolate
710 comparisons, a) full histogram and b) zoomed to <200 SNPs.